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Responses to sublethal copper exposure in two strains of *Chlamydomonas reinhardtii* (Volvocales, Chlorophyceae) in autotrophic and mixotrophic conditions

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The aim of this work was to determine the responses to sublethal copper exposure (20 and 40 μM $CuCl_2$) in two *Chlamydomonas reinhardtii* strains: the wild type *4A*$^+$ and the pleiotropic mutant *cur9*, originally selected as a copper-tolerant phenotype in mixotrophic conditions. Bioassays were performed in both autotrophic and mixotrophic media, under low irradiation (50 μmol m^{-2} s^{-1}). At the onset, Cu^{2+} concentration in culture media was estimated by MINEQL software. Oxidative stress indicators, such as changes in photosynthetic pigment ratios, catalase activity, concentration of thiobarbituric acid reactive substances (TBARS) and growth rate, demonstrated a better performance of the wild type and the mutant strain in autotrophic and mixotrophic conditions, respectively. Moderate oxidative stress was accompanied by ultrastructural changes, such as accumulation of starch and vacuoles with electronic-dense deposits and membranous structures together with mild thylakoidal and mitochondrial crest disorganization. In mixotrophic conditions isocitrate lyase (ICL) activity peaked after 72 h incubation. An inverse relation between catalase (CAT) and ICL activities was verified and proved to be a good indicator of oxidative stress correlating consistently with other characteristic features such as depression in growth rate or increase in TBARS dosage.

Key Words: *Chlamydomonas reinhardtii*, Oxidative stress, Autotrophic and mixotrophic growth, Lipid peroxidation, Catalase, Isocitrate lyase

INTRODUCTION

Industrialization and the use of pesticides and agrochemicals have driven several trace metals well beyond naturally occurring levels, and trace metal concentrations have scaled up to hazardous levels in surface and groundwater systems (Davis *et al.* 2001; Sinha *et al.* 2002). Like other trace metals, copper is persistent and can bioaccumulate within the food web, inflicting deleterious effects on algae, invertebrates and fish (Gledhill *et al.* 1997; Franklin *et al.* 2000; Pinto *et al.* 2003). In Argentinian pampas, traditionally dedicated to agriculture and raising cattle, hydrochemical analysis of three water bodies revealed that total copper mean-concentration values reached 39–89 μg l^{-1}, exceeding the maximum 13 μg l^{-1} recommended by the Environmental Protection Agency for the protection of the wildlife (Miretsky *et al.* 2004).

Copper toxicity is dependent on the metal speciation. The abundance of the divalent free copper cation, which is considered the most toxic species, depends on the physicochemical properties of the medium, such as dissolved oxygen, pH and presence of inorganic and/or organic chelators and ligands that modify the rates of association–dissociation between free and bound cation (Martin 1984).

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Oxidative stress has generally been associated with copper toxicity (Mallick & Mohn 2000; Pinto *et al.* 2003). It is still under discussion whether the generation of reactive oxygen species (ROS) is the causal agent or a mere consequence of copper toxicity. There is evidence for and against each position (Knauert & Knauer 2008 and references therein). ROS generation particularly threatens chloroplasts and mitochondria but the former have been claimed as more susceptible to trace-metal deleterious effects (Vega *et al.* 2006; Rocchetta & Küpper 2009). Copper-related oxidative damage is a light-dependent mechanism in the chloroplasts (Fischer *et al.* 2006; Knauert & Knauer 2008), where complex systems of membranes rich in polyunsaturated fatty acids are prone to lipid peroxidation (Halliwell & Gutteridge 1999).

An increase in the activity of ROS detoxifying enzymes, such as catalase, ascorbate peroxidase, superoxide dismutase and glutathione peroxidase, has been verified upon exposure to copper (Pinto *et al.* 2003; Ratkevicius *et al.* 2003; Contreras *et al.* 2005; Sabatini *et al.* 2009). Accordingly, up-regulation of genes related with antioxidant enzymes and with the removal of protein damage in copper-stressed *Chlamydomonas reinhardtii* Dangeard cells has been recently reported (Jamers *et al.* 2006).

Among the detoxifying enzymes, catalase (CAT) is capable of scavenging large quantities of H_2O_2. Therefore,

it is frequently used as an indicator of oxidative stress. Catalase in *Chlamydomonas* has some unique features: it is a mitochondrial enzyme (Kato *et al.* 1997), it contains five cysteines and it is regulated by thioredoxins (TRXs) (Lemaire *et al.* 2004).

Acetate can be used as carbon source in mixotrophic and heterotrophic cultures of *Chlamydomonas* (Harris 1989). Acetate assimilation requires isocitrate lyase, the first enzyme in the glyoxylate cycle. In contrast to plant and fungal isocitrate lyases (ICLs), ICL in *Chlamydomonas* is localized in the cytosol as is reflected by the lack of the tripeptide C-terminus sequence, necessary for peroxisomal targeting (Petridou *et al.* 1997; Schnarrenberger & Martin 2002). Light and substrate have been extensively studied as modulators of its activity (Laliberté & de la Noüe 1993; Martínez-Rivas & Vega 1993; Petridou *et al.* 1997). Recently, Rúa *et al.* (2002) proposed a regulatory mechanism through a reversible redox reaction involving cysteine residues in response to ROS, which could be mediated by proteins of the TRX family (Lemaire *et al.* 2004).

The fact that *C. reinhardtii* and its mutants can be cultured in auto, mixo and heterotrophic media allows incubation in conditions that would otherwise be lethal. This, together with the completion of nuclear and organellar DNA sequencing, has conferred model organism status to this alga (Garvey *et al.* 1991; Harris 1991), e.g. in studies of trace metal homeostasis and tolerance (Hanikenne 2003) among others.

The object of the present work was to determine the effects of copper toxicity on two strains of *C. reinhardtii* (the wild type $4A^+$ and the pleiotropic mutant *cur9*, originally selected as a copper-tolerant phenotype in mixotrophic conditions) both in autotrophic and mixotrophic media. This was done by observation of ultrastructural alterations and measurement of growth rate, chlorophyll and protein contents, lipid peroxidation and catalase level. In acetate media, ICL activity was related to catalase activation in copper-stressed cultures and correlation between both enzymatic activities served as an indicator of oxidative stress in bioassays.

MATERIAL AND METHODS

Organisms

Two strains of *C. reinhardtii* (Volvocales, Chlorophyceae) were used: the wild type $4A^+$ and the pleiotropic mutant *cur9* obtained by insertional mutagenesis with plasmid pSP124S (zeozin resistance). They were kindly provided by Dr K. Niyogi and Dr R. Dent (University of California at Berkeley). The mutant was selected as a copper-tolerant phenotype on agar plates of copper-enriched Tris-acetate-phosphate (TAP) medium. Strain stocks were maintained at 15 μmol m^{-2} s^{-1} on TAP agar plates (Harris 1989).

Chemicals

A previous screening for sublethal copper concentrations was carried out on agar plates both in high-salt (HS) and TAP media (Harris 1989) with the addition of 6.1 μM (control), 13 μM, 20 μM, 26 μM, 40 μM, 52 μM, 80 μM and 160 μM copper chloride. Analytical-grade copper chloride ($CuCl_2 \cdot 2H_2O$) was employed to obtain a stock solution of 100 mg l^{-1} in deionised water using a Sartorius 1219MP analytical balance with 0.0001 g resolution to weighing the salt. Solutions of varying concentrations were prepared through dilution of the stock with medium.

At the onset of suspension cultures, the final concentration of free divalent copper cation (Cu^{2+}) in culture media was estimated in each case using MINEQL+ 4.0 software (Table 1).

Growth conditions

Plates were inoculated with equal cell numbers of exponentially growing suspension cultures and incubated under continuous illumination (50 μmol m^{-2} s^{-1}), with growth scores being obtained periodically.

Suspension cultures were carried out a) photoautotrophically in minimal (HS) and b) photoheterotrophically in TAP media, both at 23°C with continuous agitation and illumination by cool-white fluorescent lights (50 μmol m^{-2} s^{-1}). Copper-enriched HS and TAP media were prepared with the addition of a copper solution in order to reach final nominal copper concentrations of 20 μM and 40 μM Cu. Cell density (number of cells ml^{-1}) was determined by direct counting, using an Olympus light microscope at ×400 with a 0.1 mm deep counting haemocytometer (improved Neubauer chamber). The counting of at least 25 squares of 6.25×10^{-6} ml ensured an error less than 10% (Venrick 1978). Values were expressed as average cell count ± standard deviation. Specific growth rates were calculated from the semilog plot (Wood *et al.* 2005). The ratio of palmelloid to monadoid stages was determined in random samples counting at least 100 individuals.

Ultrastructure

Cells were fixed overnight in a mixture of 1.5% p-formaldehyde + 1.5% glutaraldehyde in the corresponding filtered culture media and post-fixed for 3 h with 1% OsO_4. After dehydration in acetone series and embedding in Spurr's low viscosity resin, ultrathin sections were obtained with a diamond knife in a Reichert microtome and stained with uranyl acetate and lead citrate. Sections were observed with a Jeol 100 CX-II transmission electron microscope (Jeol Ltd., Akishima, Tokyo, Japan) at the Centro Científico Tecnológico de Bahía Blanca (CCT-BB).

Chlorophyll content

Cells from 1.5 ml cell suspension aliquots were harvested by centrifugation at 3000 rpm and thoroughly extracted with 80% acetone by vigorous vortexing. The extracts were clarified by centrifugation during 10 min at 1460 × *g*, and absorbance was read at 663 and 647 nm in a UV/Vis Shimadzu spectrophotometer. Chlorophylls *a* and *b* were determined spectrophotometrically according to the equations of Welburn (1994). Results were expressed as

Table 1. Estimation of divalent free copper cation (Cu^{2+}) and main inorganic and organic ligands in HS and TAP culture media.[1]

	Total added copper (as chloride)	Free copper Cu^{2+}	pH	Dissolved copper (%)	$CuHPO_4$	Cu-EDTA	$Cu_3(PO_4)_2$	Cu $OH(TRIS)_2$	$Cu(TRIS)_{tot}$[2]	Cu-$Acetate_{tot}$[3]
HS	6.5×10^{-6}	1.11×10^{-7}	6.56	100	4.82×10^{-6}	1.53×10^{-6}	0	0	0	0
	2.0×10^{-5}	2.13×10^{-7}	6.55	43	9.14×10^{-6}	2.07×10^{-6}	5.17×10^{-6}	0	0	0
	4.0×10^{-5}	2.45×10^{-7}	6.50	26	9.56×10^{-6}	2.19×10^{-6}	1.16×10^{-5}	0	0	0
TAP	6.5×10^{-6}	3.08×10^{-8}	6.69	100	9.81×10^{-8}	8.75×10^{-7}	0	1.72×10^{-6}	3.68×10^{-6}	1.32×10^{-7}
	2.0×10^{-5}	1.37×10^{-7}	6.69	100	4.29×10^{-7}	2.04×10^{-6}	0	5.51×10^{-6}	1.16×10^{-5}	5.86×10^{-7}
	4.0×10^{-5}	2.45×10^{-7}	6.69	100	7.67×10^{-7}	2.64×10^{-6}	0	1.14×10^{-5}	2.41×10^{-5}	1.04×10^{-6}

[1] Concentrations are expressed as moles per litre.
[2] $Cu(TRIS)_{tot}$ = Cu-TRIS + $Cu(TRIS)_2$ + $Cu(TRIS)_3$ + $Cu(TRIS)_4$.
[3] Cu-$Acetate_{tot}$ = Cu-Acetate + Cu-$Acetate_2$ + Cu-$Acetate_3$ + Cu-$Acetate_4$.

micrograms of total chlorophyll per 10^6 cells and as chlorophyll *a*/*b* ratio.

Total protein

Total protein was quantified according to Lowry *et al.* (1951), using bovine serum albumin as standard. Results were expressed as milligrams of protein per 10^6 cells.

Total extracellular carbohydrate content

Total extracellular carbohydrate in the cell-free culture media was determined by the phenol-sulphuric acid method (Dubois *et al.* 1956). Galactose was used as the standard and results were expressed as micrograms of carbohydrate per millilitre of culture media.

Lipid peroxidation

Lipid peroxides were determined as thiobarbituric acid reactive substances (TBARS) according to Vavilin *et al.* (1998). Ten-millilitre culture aliquots were used for determination. After addition of butylated hydroxytoluene [final concentration, 0.01% (w/v)] to terminate lipid peroxidation chain reactions, cells were resuspended in freshly prepared trichloroacetic acid (with and without thiobarbituric acid). Following incubation at 95°C for 30 min, samples were cooled to room temperature. TBARS were determined by absorbance at 532 nm with a correction for nonspecific absorbance at 440 and 600 nm (Hodges *et al.* 1999), using a molar extinction coefficient for the thiobarbituric acid–malondialdehyde complex of 155 mM^{-1} cm^{-1}. Results were expressed as micromoles of TBARS per 10^6 cells.

Enzymatic activity assays

Cells harvested by centrifugation at 1460 × g at 4°C were resuspended in 5 ml of 20 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM mercaptoethanol. Cell rupture was achieved by ultrasonication, employing three 10-s successive cycles with a maximum frequency of 20 KHz with 50 s interval. All procedures were performed at 4°C. The sonicated cells were centrifuged at 15,450 × *g* for 20 min to separate the supernatants for further analyses.

CAT (EC 1.11.1.6) activity was measured with H_2O_2 as substrate and calculated using an extinction coefficient for H_2O_2 of 40 M^{-1} cm^{-2} (Aebi 1984). The decay of peroxide was monitored during 30 s at 240 nm, in a reaction mixture containing 40 mM potassium phosphate (pH 7.5) and 10 mM H_2O_2 added at the onset of the reaction. One CAT unit was defined as the enzyme amount that transforms 1 mmol of H_2O_2 per minute. Enzymatic activity was expressed as CAT units per milligram of protein.

ICL (EC 4.1.3.1) activity was measured using threo-D_sL_s-isocitric acid as substrate. Glyoxylate accumulation was calculated as the formation of the hydrazone in the presence of phenylhydrazine-HCl (Cooper & Beavers 1969) by reading absorbance at 324 nm (extinction coefficient 14.63 × 10^3 M^{-1} cm^{-2}). The reaction mixture (1 ml) contained 87 mM potassium phosphate buffer (pH 6.9), 4.6 mM mercaptoethanol, 87 mM $MgCl_2$, 30–40 µl of cellular

homogenate and 10 mM phenylhydrazine. At the onset, 13 mmol of D_sL_s-isocitric acid were added, and the reaction was monitored for 5 min. One ICL unit was defined as the enzyme amount that catalyzes the formation of 1 μmol of glyoxylate phenylhydrazone per minute at pH 6.9 and 30°C. Enzymatic activity was expressed as ICL units per mg protein.

Statistical analysis

The results of three replicates from different treatments were compared statistically by one-way analysis of variance (ANOVA) followed by a Dunnet *post hoc* test. The suppositions of normality and homogeneity of variances were tested with Lillieford and Bartlett tests, respectively (Sokal & Rohlf 1995). The relationship between the parameters of ICL activity and CAT activity were analyzed by regression analysis (Sokal & Rohlf 1995). Graph Pad Prism 3 software was used for statistical analysis.

RESULTS

Plaque bioassays

Algal strains showed a different behaviour in plaque assays depending on the media. Both strains developed colonies after 96 h of incubation in both media up to 40 μM $CuCl_2$. In autotrophic conditions, the mutant strain *cur9* did not produce colonies in HS medium augmented with 52 μM $CuCl_2$, while the wild strain did. On the contrary, in TAP medium augmented with 52 μM $CuCl_2$, only the mutant strain grew. Colonies in copper-enriched media always showed bleaching with respect to the controls. According to these results, 20 and 40 μM $CuCl_2$ were selected for suspension bioassays.

Estimated free divalent cation concentration

Nonlethal concentrations for suspension cultures were analyzed with MINEQL software, aimed at submitting the algae to similar free divalent copper cation concentrations in the media at the onset of the bioassay. Table 1 shows that pH decreased slightly (from 6.56 to 6.50) with the increase in copper concentration in HS mineral medium, while it remained unaltered in TAP medium (6.69). Furthermore, only 43% and 26% of copper compounds were soluble in 20 and 40 μM $CuCl_2$ HS media, respectively, while the totality of copper species was soluble in TAP. Note that in HS, EDTA and phosphate bind copper, while Tris gains importance as a chelator in TAP medium, especially when increasing copper concentration to 20 and 40 μM $CuCl_2$. As pointed out by Kiefer *et al.* (1997), at higher copper concentration carboxylate groups (e.g. in acetate) are relevant binding sites but at lower concentrations higher affinity ligands such as N-donor atoms (e.g. EDTA and TRIS) are preferred. The free divalent cation (Cu^{2+}) for 40 μM $CuCl_2$ HS and 40 μM $CuCl_2$ TAP media were identical (2.45×10^{-7} M). The absence of organic ligands for copper in 20 μM $CuCl_2$ HS medium rendered a higher free divalent cation concentration in the inorganic medium (2.13×10^{-7} M vs 1.37×10^{-7}M).

Table 2. Specific growth rates (k) of *Chlamydomonas reinhardtii* $4A^+$ and *cur9* in control and copper enriched cultures in HS and TAP media.[1]

Strain	Culture media	K (day^{-1})
$4A^+$	HS 6.1 μM $CuCl_2$	0.940 ± 0.029
	HS 20 μM $CuCl_2$	0.817 ± 0.023
	HS 40 μM $CuCl_2$	0.730 ± 0.152
cur9	HS 6.1 μM $CuCl_2$	0.906 ± 0.041
	HS 20 μM $CuCl_2$	0.535 ± 0.077**
	HS 40 μM $CuCl_2$	0.494 ± 0.061**
$4A^+$	TAP 6.1 μM $CuCl_2$	1.923 ± 0.095
	TAP 20 μM $CuCl_2$	1.834 ± 0.021
	TAP 40 μM $CuCl_2$	1.274 ± 0.160**
cur9	TAP 6.1 μM $CuCl_2$	1.499 ± 0.132
	TAP 20 μM $CuCl_2$	1.376 ± 0.123
	TAP 40 μM $CuCl_2$	1.304 ± 0.111

[1] Significant differences between control and treatments are indicated by asterisks: $^{**}P < 0.01$, $n = 3$.

Culture growth rate

In HS medium, specific growth rates (Table 2) were negatively affected by copper for the mutant strain ($P < 0.01$ for *cur9* 20 μM $CuCl_2$ HS and 40 μM $CuCl_2$ HS). Incubation in the presence of acetate reversed the inhibitory effect of copper on mutant growth rates. In fact, no significant differences were found with respect to the control. On the other hand, 40 μM $CuCl_2$ TAP medium caused a significant reduction in growth rate in the wild type ($P < 0.001$).

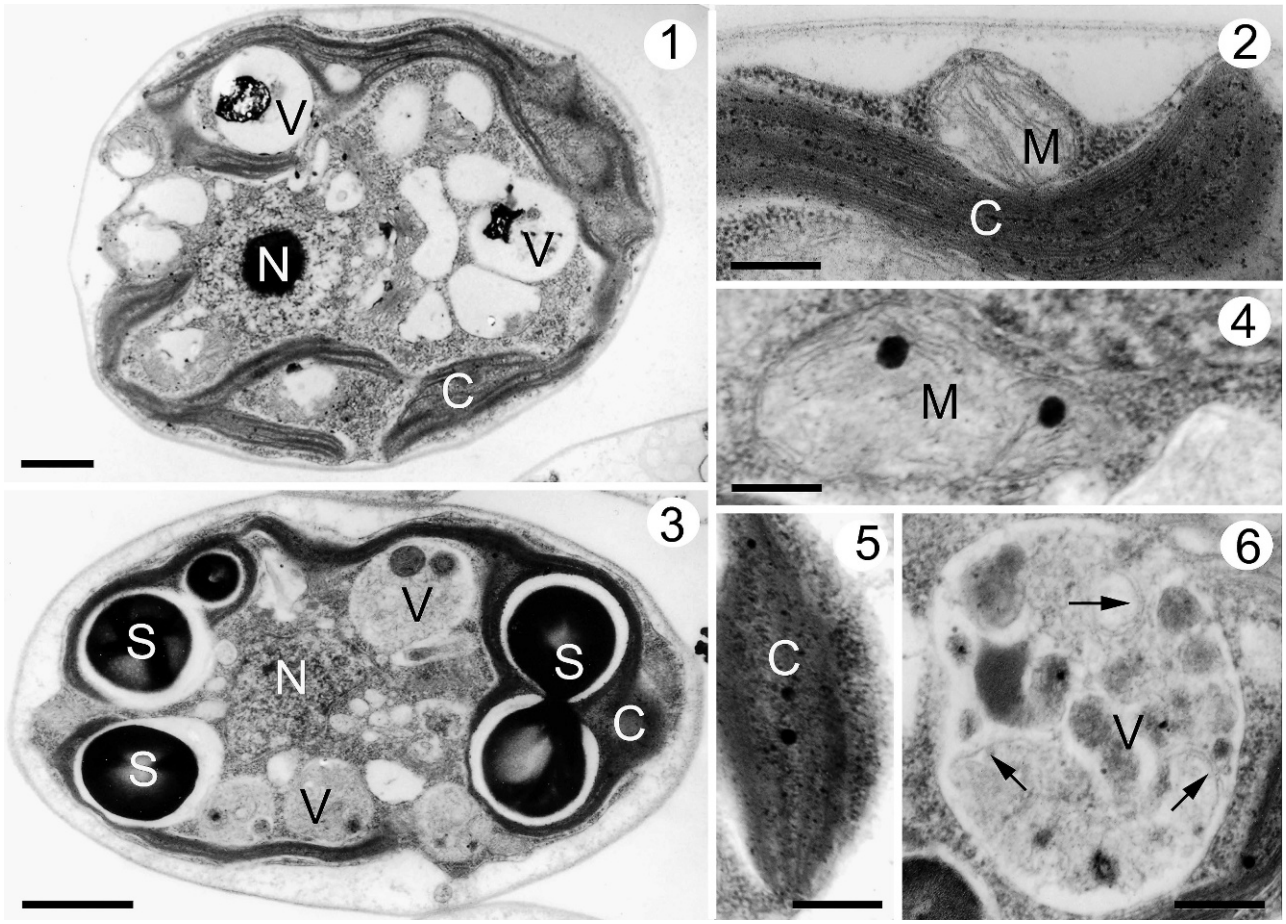
Additional morphological observations indicated that four-celled palmelloid stages only appeared in HS medium in a low proportion (10%). This frequency tended to augment with the increase in copper concentration, especially for the wild type $4A^+$. Palmelloids were first registered in the wild type at 96 h; for the mutant strain, at 144 h.

Ultrastructural observations

Control cells presented the typical ultrastructure of the genus *Chlamydomonas* (Figs 1, 2). In both strains, cells grown in the presence of copper showed no significant changes in their ellipsoidal shape (Figs 1, 3). However, some changes were observed in their ultrastructure. Copper caused incipient thylakoid disorganization and higher content starch accumulation (Figs 3, 5). Mitochondria presented irregular shape and crest disorganization and loss (Fig. 4). The vacuoles contained electron-dense deposits and membrane remnants [Figs 3, 6 (arrows)].

Pigment composition

Average total chlorophyll content in $4A^+$ controls showed no significant differences between autotrophic (2.312 ± 0.36 μg per 10^6 cells) and mixotrophic conditions (1.90 ± 0.07 μg per 10^6 cells) (Fig. 7A, B). Instead, the addition of acetate to the media led to an average 59% decrease in total



Figs 1–6. Transmission electron micrographs of *Chlamydomonas reinhardtti* mutant strain *cur9*.
Figs 1–2. Control cells in TAP medium.
Fig. 1. General view of a cell with an anterior nucleus, parietal chloroplast and vacuoles with electron-dense inclusions. Scale bar = 1.5 μm.
Fig. 2. Detail of a cell portion showing the parallel thylakoids in the chloroplast and a mitochondrion. Scale bar = 5 μm.
Figs 3–6. Cells after 120 h exposure to 40 μM copper.
Fig. 3. General view of a cell with numerous starch grains. Scale bar = 1.5 μm.
Fig. 4. Detail of a mitochondrion with irregular shape and crest disorganization and loss. Scale bar = 5 μm.
Fig. 5. Detail of a chloroplast with disorganized thylakoids. Scale bar = 5 μm.
Fig. 6. Detail of a vacuole with electron-dense inclusions and membranes inside (arrows). Scale bar = 5 μm. C, chloroplast; M, mitochondrion; N, nucleus; S, starch; V, vacuole.

chlorophyll for control cultures of *cur9* (2.18 ± 0.26 μg per 10^6 cells vs 1.29 ± 0.25 μg per 10^6 cells) (Fig. 7A, B).

Throughout the entire bioassay for *cur9*, 40 μM $CuCl_2$ HS medium induced significant bleaching ($P < 0.01$ and $P < 0.001$), while significant decolouring ($P < 0.05$) only appeared at the end point in the wild type (Fig. 7A). On the contrary, under mixotrophic conditions significant copper-enhanced bleaching ($P < 0.05$) was detected for the wild type in both 20 and 40 μM $CuCl_2$ TAP media (Fig. 7B). For mutant strain, bleaching was observed in 40 μM $CuCl_2$ ($P < 0.01$ and $P < 0.001$) (Figure 7B).

In addition, the chlorophyll *a*/*b* ratio was calculated. In HS medium, the only significant increase ($P < 0.05$ and $P < 0.01$) was registered for the wild type at the initial stages of growth (48 h) (Fig. 8A). In the presence of acetate, this ratio showed significant increases ($P < 0.05$, $P < 0.01$ and $P < 0.001$) for both strains; ratios increased with higher metal concentration (Fig. 8B). Towards the end of the bioassay (96–120 h) differences with the controls were less evident or even not significant (see 20 μM $CuCl_2$ TAP medium in Fig. 8B).

Lipid peroxidation

A significant rise in TBARS was registered throughout the bioassay for both strains in 40 μM $CuCl_2$ HS medium ($P < 0.01$ and $P < 0.001$) (Fig. 9A). Exposure to 20 μM $CuCl_2$ in autotrophic conditions caused an earlier significant rise in TBARS for *cur9* (from 144 to 264 h), while the same occurred in a later stage for $4A^+$ (from 216 to 264 h) (Fig. 9A). In TAP medium, response to 40 μM $CuCl_2$ differed for both strains: in the wild type oxidative damage appeared early (at 24 h), while in *cur9* it was detected from 72 h onwards (Fig. 9B) ($P < 0.001$). Instead, upon exposure to 20 μM $CuCl_2$ TAP medium, significant oxidative damage with respect to the controls was observed for both strains from 72 h onwards ($P < 0.05$ and $P < 0.001$), even when free-cation concentration was half the amount in HS

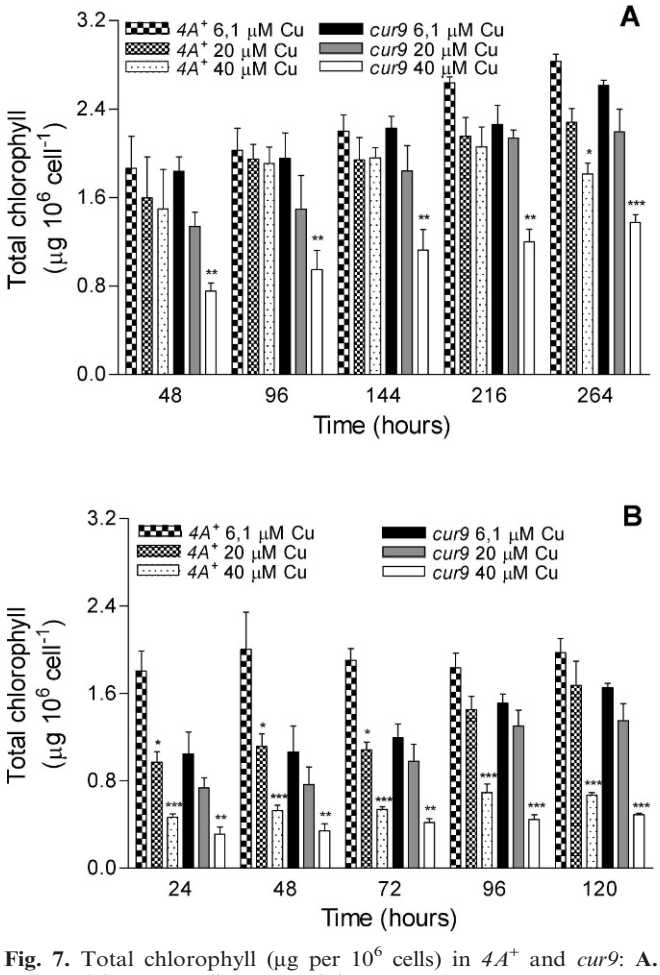


Fig. 7. Total chlorophyll (µg per 10^6 cells) in $4A^+$ and *cur9*: **A.** Cultured in HS medium containing 20 and 40 µM copper over 264 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. **B.** Cultured in TAP medium containing 20 and 40 µM copper over 120 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

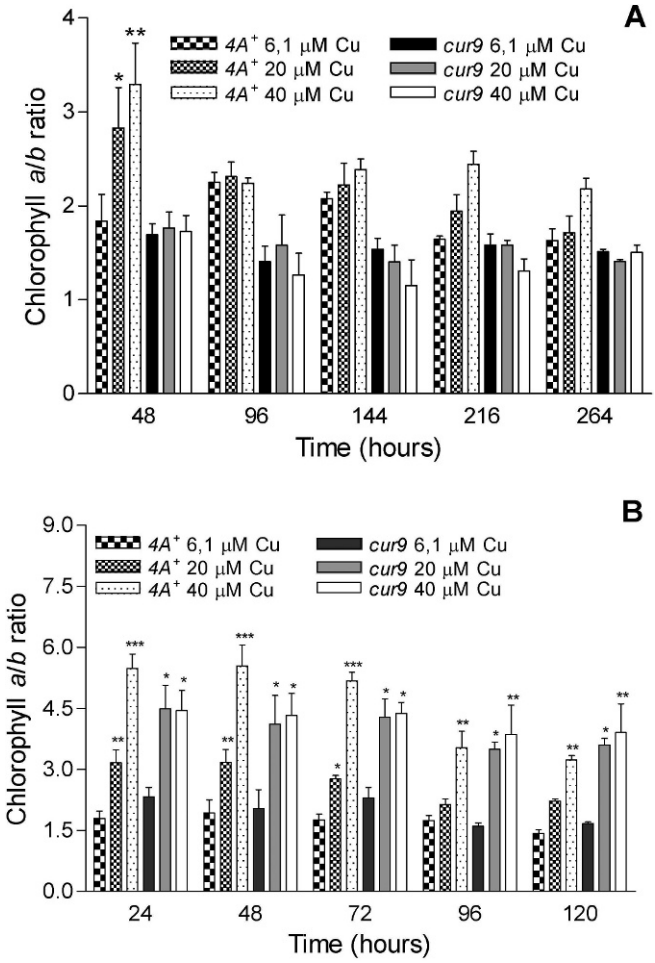


Fig. 8. Chlorophyll *a*/*b* ratio in $4A^+$ and *cur9*: **A.** Cultured in HS medium containing 20 and 40 µM copper over 264 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. **B.** Cultured in TAP medium containing 20 and 40 µM copper over 120 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

medium for the same nominal copper chloride total. Nevertheless, TBARS dosage was always higher in mineral media than in the TAP (cf. Fig. 9A and B), especially for the mutant strain.

Protein content

Copper exposure caused significant increases in protein content for the mutant strain throughout the bioassay in HS medium, while the wild type only showed significant differences at 48 h (Fig. 10A). Incubation in 40 µM $CuCl_2$ TAP medium induced a significant protein increase up to 96 h only for $4A^+$ (Fig. 10B).

Extracellular carbohydrates

Accumulation of extracellular carbohydrates increased towards the stationary phase in both autotrophic and mixotrophic conditions, being always higher in HS medium (Fig. 11A, B). The deleterious effect of copper on sugar secretion differed with culture conditions and strain. The inhibitory effect of the metal was enhanced in mixotrophic and autotrophic conditions for the wild type and the mutant strain, respectively ($P < 0.01$ and $P < 0.001$) (Fig. 11A, B).

Catalase activity

Under autotrophic conditions (20 and 40 µM $CuCl_2$ HS media), copper led to a significant increase ($P < 0.05$, $P < 0.01$ and $P < 0.001$) in catalase activity for strain $4A^+$ (Fig. 12A). In the presence of acetate (20 and 40 µM $CuCl_2$ TAP media), both strains showed significant increase in CAT activity ($P < 0.05$, $P < 0.01$ and $P < 0.001$), always being higher for *cur9* (Fig. 12B).

Isocitrate lyase activity

In control mixotrophic cultures of $4A^+$ and *cur9*, ICL activity peaked at 72 h after the onset of the incubation

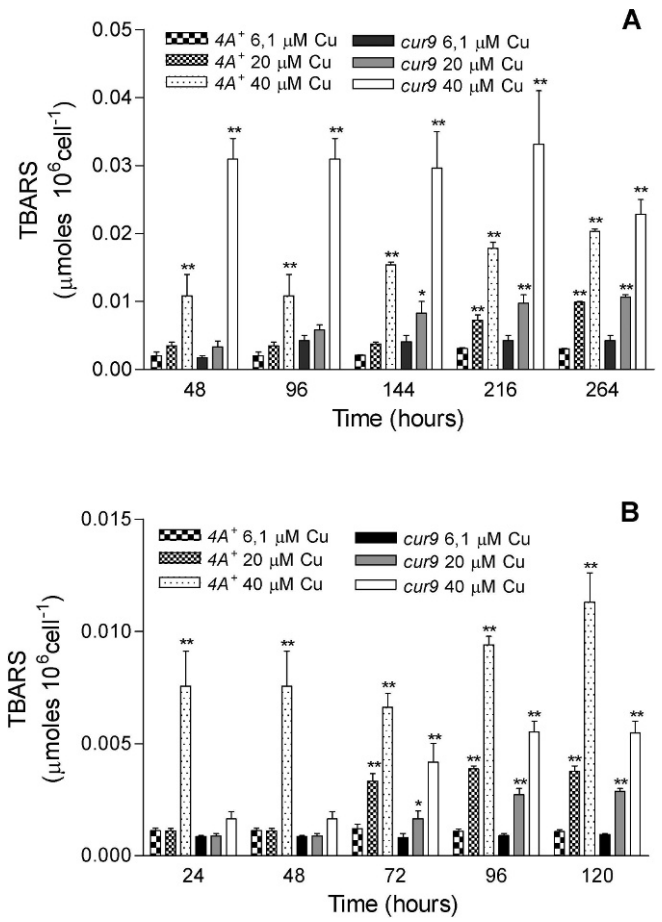


Fig. 9. TBARS content (µmol per 10^6 cells) in $4A^+$ and *cur9*: **A.** Cultured in HS medium containing 20 and 40 µM copper over 264 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.01$ and $**P < 0.001$. **B.** Cultured in TAP medium containing 20 and 40 µM copper over 120 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.01$ and $**P < 0.001$.

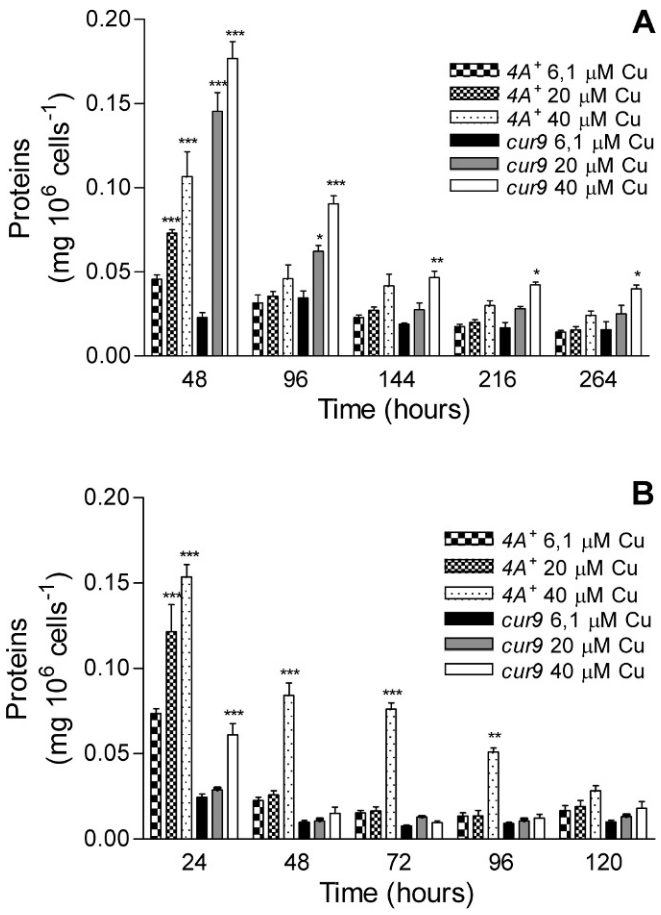


Fig. 10. Protein content (µg protein per 10^6 cells) in $4A^+$ and *cur9*: **A.** Cultured in HS medium containing 20 and 40 µM copper over 264 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by an asterisk: $*P < 0.05$. **B.** Cultured in TAP medium containing 20 and 40 µM copper over 120 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.01$ and $**P < 0.001$.

(Fig. 13). Mutant strain showed at least seven times higher activity than the wild type (cf. controls, Fig. 13). Acetate assimilation was significantly inhibited for both strains ($P < 0.05$, $P < 0.01$ and $P < 0.001$) in 40 µM $CuCl_2$ TAP medium. Additionally, *cur 9* was also inhibited ($P < 0.05$ and $P < 0.01$) in 20µM $CuCl_2$ TAP medium.

A significant negative correlation was found between CAT and ICL activities at 72 h, indicating that high detoxifying enzymatic activity is related to lower acetate assimilation in both strains (Fig. 14). Despite the smoother slope in the regression line for $4A^+$ with respect to *cur9*, an inverse correlation between the two enzymatic activities was still evident.

DISCUSSION

Suspension and plaque bioassays revealed low resistance to copper exposure in both strains, especially when comparing our data with the nominal metal concentrations used by Takamura *et al.* (1989) in bioassays with other Chlorophyta isolated from copper-polluted sites. The copper effect on specific growth rates for each strain was culture medium-dependant: growth rates diminished significantly for the wild type only in TAP medium, while for the mutant strain they did so in HS medium.

Detoxification by exclusion of trace metals is accomplished through different cellular mechanisms: adsorption to extracellular ligands, precipitation, reduction and active transport (Corradi *et al.* 1998; Cobbett *et al.* 2003; Pinto *et al.* 2003; Perales-Vela *et al.* 2006; Sabatini *et al.* 2009). Extracellular exclusion by carbohydrate secretion proved ineffective as a protective mechanism in this bioassay. In fact, metal had a deleterious effect on carbohydrate liberation. Instead, intracellular starch accumulation was observed together with mild thylakoid disorganization. Stromal starch accumulation in *Chlamydomonas* has been attributed to the lack of physical constraint due to poor thylakoid membrane stacking (Inwood *et al.* 2008). Metal driven increases in starch content and/or granule size and the reduction in mitochondrial size have been reported for *Chlamydomonas acidophyla* (Nishikawa *et al.* 2003). Im-

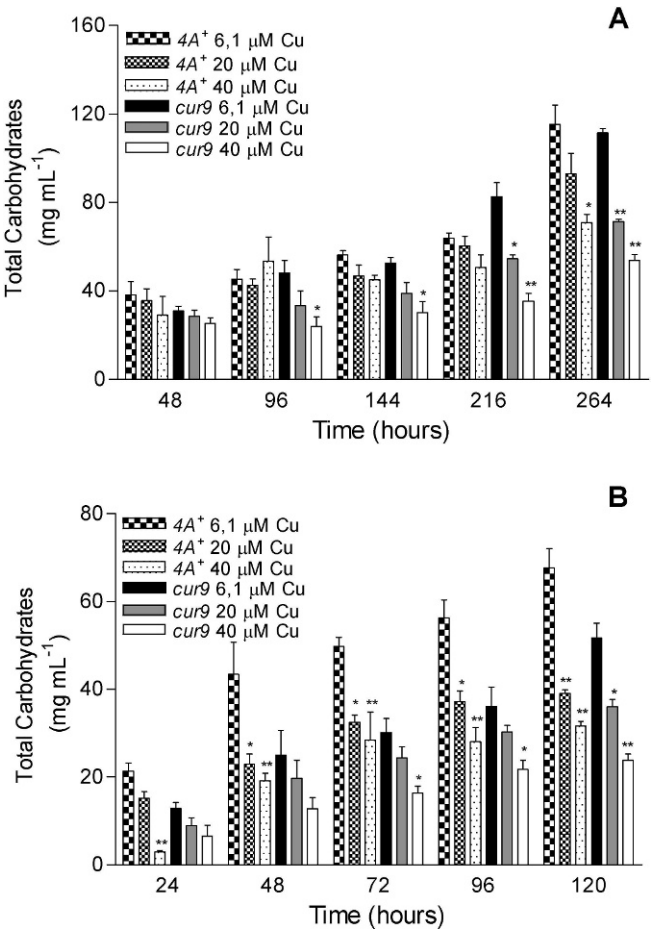


Fig. 11. Total carbohydrates (μg ml^{-1}) in $4A^+$ and *cur9:* **A.** Cultured in HS medium containing 20 and 40 μM copper over 264 h. Data are expressed as means ± SD (n = 3). Significant differences between control and treatments are indicated by asterisks: *$P < 0.01$ and **$P < 0.001$. **B.** Cultured in TAP medium containing 20 and 40 μM copper over 120 h. Data are expressed as means ± SD (n = 3). Significant differences between control and treatments are indicated by asterisks: *$P < 0.01$ and **$P < 0.001$.

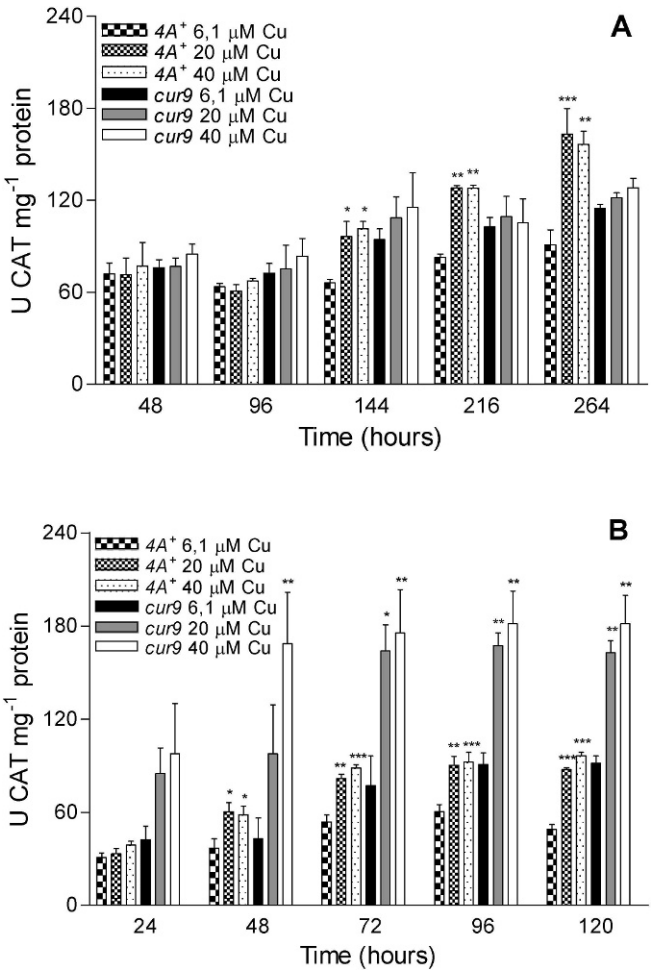


Fig. 12. Catalase activity (U CAT mg^{-1} protein) in $4A^+$ and *cur9*: **A.** Cultured in HS medium containing 20 and 40 μM copper over 264 h. Data are expressed as means ± SD (n = 3). Significant differences between control and treatments are indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **B.** Cultured in TAP medium containing 20 and 40 μM copper over 120 h. Data are expressed as means ± SD (n = 3). Significant differences between control and treatments are indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

paired utilization of starch as a respiratory substrate or delayed cell division may result in storage polysaccharide accumulation (Libessart *et al.* 1995). On the other hand, Ballan-Dufrançais *et al.* (1991) and Nassiri *et al.* (1996) claimed that copper-contaminated cells of *Tetraselmis suecica* showed starch depletion as a consequence of impaired photosynthesis.

The proliferation of intracellular membrane whorls in cyanobacteria (Rachlin *et al.* 1982) and metal detoxification within vacuoles, whether associated with membranous structures or formation of electron-dense deposits, have also been reported (Nassiri *et al.* 1996; Nishikawa *et al.* 2003; Perales-Vela *et al.* 2006; Rochetta *et al.* 2007). Garvey *et al.* (1991) highlighted a tendency to deflagellation when subjecting *Chlamydomonas* cells to trace metal stress. A similar effect was informed for *T. suecica* (Ballan-Dufrançais *et al.* 1991; Nassiri *et al.* 1996). Since palmelloid stages provide an additional extracellular covering contributing to extracellular metal immobilization, the observed tendency for palmelloid formation in HS medium suggests a protective role of this mechanism in this strain.

In microalgae, respiratory activity is less sensitive than photosynthesis to copper and to other trace metals (Vega *et al.* 2006; Rocchetta & Küpper 2009). In fact, metal targeting at the photosynthetic apparatus leading to impaired biomass production is the basis for copper application as an algaecide (Murray-Gulde *et al.* 2002). Even though photosynthetic efficiency was not measured in the present work, chlorophyll quantification can provide an indirect evidence of photosystem (PS) status, since chlorophyll *a*/*b* ratio reflects changes in the size and composition of PSI and PSII (Smith *et al.* 1990; Melis 1991). Though in HS medium the chlorophyll *a*/*b* ratio does not indicate changes in PS status (except for the mentioned initial stage in the wild type), there is a significant reduction in the specific growth rate for *cur9*. With mild bleaching, it has been previously reported that lipid peroxidation may not be

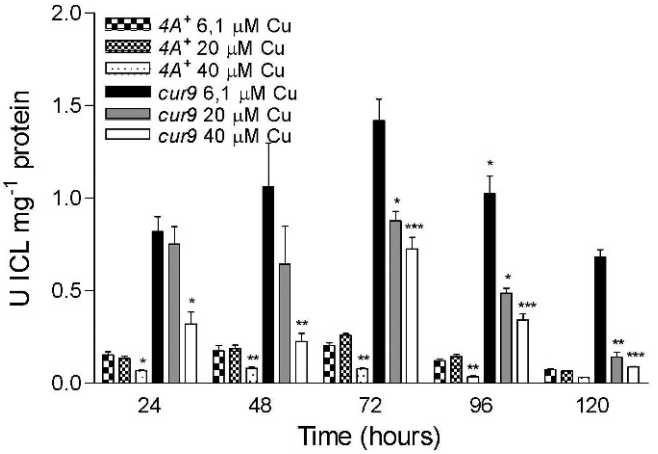


Fig. 13. Isocitrate lyase activity (U ICL mg^{-1} protein) in $4A^+$ and *cur9* cultured in TAP medium containing 20 and 40 µM copper over 120 h. Data are expressed as means ± SD ($n = 3$). Significant differences between control and treatments are indicated by asterisks: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

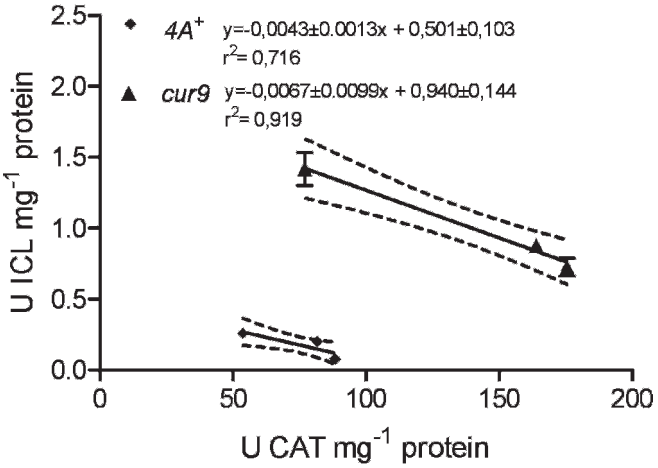


Fig. 14. Regression between isocitrate lyase activity and catalase activity in *cur9* and $4A^+$ ($n = 3$).

severe enough to affect PS architecture (Rocchetta & Küpper 2009). Considering the increase in TBARS content as an oxidative stress indicator, the consequences of copper exposure in autotrophic conditions for *cur9* would be an energy investment in overcoming stress at the expense of growth. Thus, growth rate reduction for the mutant strain in autotrophic conditions proved to be a more sensitive indicator of copper toxicity than the chlorophyll *a*/*b* ratio. Similar results were reported for *Scenedesmus incrassatulus* by Perales-Vela *et al.* (2007).

In acetate-supplied cultures, total chlorophyll content was always lower than in mineral media, as has been previously reported (Fischer *et al.* 2006). As these authors point out, under nonoxidative stress conditions, TAP-grown cells use acetate as the main carbon source, and cyclic electron flow in photosynthesis for ATP production is favoured over linear flow from water to $NADP^+$, associated with oxygen evolution and ATP production through PSII (Turpin 1991, Fischer *et al.* 2006). To cope with the competitive requirements for ATP of the Calvin and glyoxylate cycles, green algae respond by decreasing the ratio of PSII to PSI; this reduction in PSII abundance being achieved by down-regulation of D1 expression (Heifetz *et al.* 2000; Kovacs *et al.* 2000). ICL activity peaked at 72 h, always being higher for *cur 9* (five times higher for control and 20 µM $CuCl_2$ and 10 times higher for 40 µM $CuCl_2$). Accordingly, higher ICL activity in *cur9* is accompanied by an increase in chlorophyll *a*/*b* ratio in controls with respect to autotrophic conditions. The addition of copper to TAP always produced an increase in chlorophyll *a*/*b* ratio in both strains, indicating that the metal cation enhanced the alteration of the relative abundance of the photosystems. Formation of pheophytin and Cu^{2+} substitution of Mg^{2+} in chlorophyll molecules in the light-harvesting complex associated with PSII under low irradiances, as used in the present work, could also explain the changes in the chlorophyll *a*/*b* ratio (Küpper *et al.* 2002; Rodríguez *et al.* 2007; Rocchetta & Küpper 2009).

The fact that TRXs play a key role in oxidative stress responses, either through redox signalling or as electron donors (Dietz 2003), has provided a new insight on the regulation of several enzyme activities in *Chlamydomonas* (Rúa *et al.* 2002; Lemaire *et al.* 2004). In particular, the observations that reduced TRX inhibited catalase activity on the one hand, while on the other hand it activated isocitrate lyase (Lemaire *et al.* 2004) were revealing. In the present study, under metal-induced oxidative stress in mixotrophic conditions, we would expect a higher proportion of oxidised TRX. Thus, the detoxifying enzyme (CAT) would be activated while acetate assimilation in the glyoxylate cycle would be inhibited, in agreement with the observed negative correlation between both enzymatic activities. ICL activity in strain *cur9* is higher than that of the wild type in the controls. When comparing the decay range in enzymatic activity upon copper exposure, it is notably steeper for the mutant. This could be due to strain differences with respect to ROS detoxifying capacity of CAT and/or redox state perception in both CAT and ICL. The higher copper tolerance exhibited by *cur9* in mixotrophic conditions suggests a higher intrinsic ICL activity rather than higher CAT detoxifying capacity. Accordingly, when *cur9* was exposed to copper in autotrophic conditions, it exhibited a lower increase in CAT activity and higher lipid peroxidation than the wild type.

CONCLUSIONS

Through the comparison of results obtained from algal incubation in autotrophic and mixotrophic conditions, we conclude that under low irradiance, oxidative stress indicators (such as changes in photosystem status, CAT activity, TBARS content and specific growth rate) demonstrated that the wild type had a better performance in autotrophic conditions, while for the mutant strain this behaviour was obtained in mixotrophic conditions.

The correlation between ICL and CAT activity (taken at the peak of the acetate-assimilating enzyme activity) is a good indicator of oxidative stress in bioassays of *C. reinhardtii* incubated in TAP medium at low irradiances. The inverse relation between both enzymatic activities is in

agreement with other characteristic features of growth under oxidative stress, such as depression in growth rate or increase in TBARS dosage. Additionally, incubation in mixotrophic conditions provides the advantage of shorter duration of bioassays and a complete solubilization of copper compounds.

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